Exhibit A

Production of fully human antibodies by transgenic mice

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The ability to produce a diverse repertoire of fully human monoclonal antibodies may have significant applications to human therapy. One of the most promising approaches to the production of therapeutic human monoclonal antibodies is the creation of a mouse strain engineered to produce a large repertoire of human antibodies in the absence of mouse antibodies. Recently, such mice have been generated by introducing segments of human immunoglobulin loci into the germlines of mice deficient in mouse antibody production as a result of gene targeting. These mice produce significant levels of fully human antibodies with a diverse adult-like repertoire and, upon immunization with antigens, generate antigen-specific fully human monoclonal antibodies. Such strains of mice may provide the optimal source for producing human monoclonal antibodies with high affinity and specificity against a broad spectrum of antigens, including human antigens.

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Introduction

Since their discovery in 1975 [1], monoclonal antibodies have held great promise for the treatment of human diseases such as cancer, autoimmune disorders, and viral infections. Mouse monoclonal antibodies, with high affinity and specificity to desired targets, are relatively easy to produce, but are severely restricted for therapeutic use because of their immunogenicity in humans and the resulting reduction in their efficacy and safety. Such limitations could be overcome by the availability of fully human monoclonal antibodies that would permit repeated administration without immunogenic and allergic responses.

Application of the hybridoma methodology to produce antigen-specific human monoclonal antibodies from immunized human B cells has been limited by the availability of human B cells expressing high-affinity antibodies of the desired antigen specificity and by the difficulty of achieving their immortalization (for a review, see [2]). Genetic engineering of mouse monoclonal antibodies to generate chimeric [3] or humanized [4] antibodies with reduced immunogenicity requires case-by-case design and engineering of each individual antibody, which still retains mouse sequences. The generation of large human immunoglobulin (Ig) gene combinatorial libraries opened the way for cloning of antigen-specific fully human antibodies [5,6]. Yet, as the availability of B cells from naturally immunized individuals is sporadic and limited, and protocols for *in vitro* immunization of human B cells are yet to be established, the generation of useful human antibodies by this technology is likely to require extensive *in vitro* manipulation.

The difficulties encountered with *in vitro* production of human, or partially human, antibodies of therapeutic value, have redirected attention to the mouse as an ideal expression system for generation and selection of high-affinity authentic human antibodies. This was attempted by engraftment of human B cells or human Ig genes into mice.

Engraftment of immunodeficient mice with human lymphoid cells has resulted in the production of significant levels of human immunoglobulins and in the generation of antigen-specific human antibody responses to recall antigens as well as to some neoantigens [7,8]. Even so, this approach is likely to be limited by the restricted response of the engrafted cells to many self antigens, which may constitute an important class of therapeutic targets, and by the problems associated with human B-cell immortalization.

These limitations can be overcome by generating mouse strains engineered with unrearranged human Ig genes to produce a large array of human antibodies in the absence of mouse antibodies. Such a mouse would lack immunological tolerance to human proteins, and thus readily yield antibodies to these molecules. Therefore, they could be used to generate fully human antibodies, with high affinity and specificity, and directed

Abbreviations

CDR—complementarity-determining region; **DI**—double-inactivated; **ES**—embryonic-stem; **Ig**—immunoglobulin; **YAC**—yeast artificial chromosome.

against a broad spectrum of antigens. Furthermore, antigen-specific human monoclonal antibodies could be easily generated from these mice using standard mouse hybridoma technology.

In this review, I describe the generation and characterization of mice engineered with human Ig fragments. I then discuss how these mice may be utilized as a source for producing antigen-specific fully human monoclonal anitbodies.

Generating transgenic mice with human immunoglobulin loci

The creation of a mouse strain producing human antibodies in the absence of mouse antibodies involved two major genetic manipulations of the mouse genome: inactivation of mouse Ig genes, and stable introduction of cloned human Ig loci (see Fig. 1). The genes encoding human Ig heavy and κ light chains each span over 1.5 Mb on chromosomes 14 and 2, respectively [9,10]. In their germline configuration, these loci consist of a large diversity of segments encoding the variable (V), diversity (D) and joining (J) genes that comprise the variable domains, and the segments that encode the constant (C) domains. Antibody diversity is primarily generated from the combinatorial rearrangement between the different V segments (95 V_H genes and 76 V_κ genes), D_H segments (~30) and J genes (six J_H and five J_κ) which span most of the heavy and κ light-chain loci [10,11]. These loci also contain the interspersed regulatory elements, which control antibody expression, allelic exclusion, class switching and affinity maturation [12].

The reproduction of human antibody responses in mice is likely to be achieved upon the introduction of a large portion of the human Ig loci, thus preserving the large variable gene diversity as well as the proper regulation of antibody production and expression. This approach, which demands the ability both to clone and reconstruct large portions of the human Ig loci in their germline configuration and to introduce them in intact form into the mouse germline, is the essence of one of the two strategies for creating human antibody-producing mice [13••].

Cloning of large fragments of human heavy- and light-chain loci was achieved by the yeast artificial chromosome (YAC) technology which permits the isolation and genetic manipulation of DNA fragments megabases in size [14]. The technology for introduction of megabase-sized molecules into embryonic stem (ES) cells was established by the fusion of YAC-containing yeast spheroplasts and ES cells [15].

YACs containing variable and constant sequences from the human heavy-chain and light κ chain loci, identified from the Washington University human YAC library, have been characterized and determined to be in an intact germline configuration [13••,16•]. The 245 kb human heavy-chain YAC (yH1) contained the μ and δ constant regions, all six functional J_H genes, the entire D region, the intronic enhancer, and five V genes (V_{VI}, V_{I-2}, V_{I-3}, V_{IV-4} and V_{II-5}) from four human V_H families. The 190 kb human κ YAC (yK1) contains the κ deleting element (Kde), the intronic and 3′ enhancers, the C_κ constant region, all five J_κ genes, and the three V_κ variable genes in the B cluster. The introduction of human Ig YACs into the mouse ES cells via fusion yielded a high frequency of ES clones bearing a single intact YAC [13••,16•]. The integrated YACs were transmitted faithfully through the mouse germline to generate mouse strains containing yH1 and yK1 which were bred with mice deficient in mouse antibody production [13••].

Elimination of mouse antibody production was achieved by inactivation of mouse Ig genes in ES cells, using gene-targeting technology to delete crucial *cis*-acting sequences involved in the process of mouse Ig gene rearrangement and expression. The J_H region, which is involved in the critical first Ig rearrangement steps, and the single C_κ region were deleted from the mouse heavy chain and κ light chain, respectively [13••,17]. Targeted ES cell clones were used to generate a mouse colony of mice homozygous for each of the J_H or C_κ deletions. Crossbreeding of these two mouse strains generated double-inactivated (DI) mice, homozygous for both mutations, in which production of antibodies and, therefore, B-cell development, is completely blocked [13••]. DI mice, however, are capable of antibody rearrangement and expression and thus are a suitable host for reconstitution with unrearranged human Ig genes. Breeding of DI mice with yH1- and yK1-containing mice generated xenomouse strains producing human antibodies (Fig. 1) [13••].

Another approach taken to generate human antibody producing mice utilizes human Ig minigenes, which are constructed by stitching together non-contiguous genomic segments of variable and constant regions from the human Ig loci [18–20,21••]. Transgenic mice producing fully human antibodies have been generated from two sets of human heavy-chain and κ-chain minigenes [20,21••]. The largest heavy-chain transgene constructed (HC2, 80 kb) contained four functional V segments ($V_{H4.21}$, V_{H51p1}, V_{H56p1} and V_{H251}), 15 D segments, six J segments, the μ- and γ1-coding exons and their respective switch regions, as well as the Jμ intronic enhancer and the rat 3′ enhancer. The largest light-chain transgene constructed (KCo4, 43 kb) contained four functional V genes ($V_{k65.3}$, $V_{k65.5}$, $V_{k65.8}$ and $V_{k65.15}$), five J segments, the C_κ exon and the intronic and downstream enhancer elements. Microinjection of these constructs into mouse pronuclei generated transgenic mice with human Ig miniloci integrated primarily in multiple copies. These mice were bred with heavy-chain and κ light chain inactivated

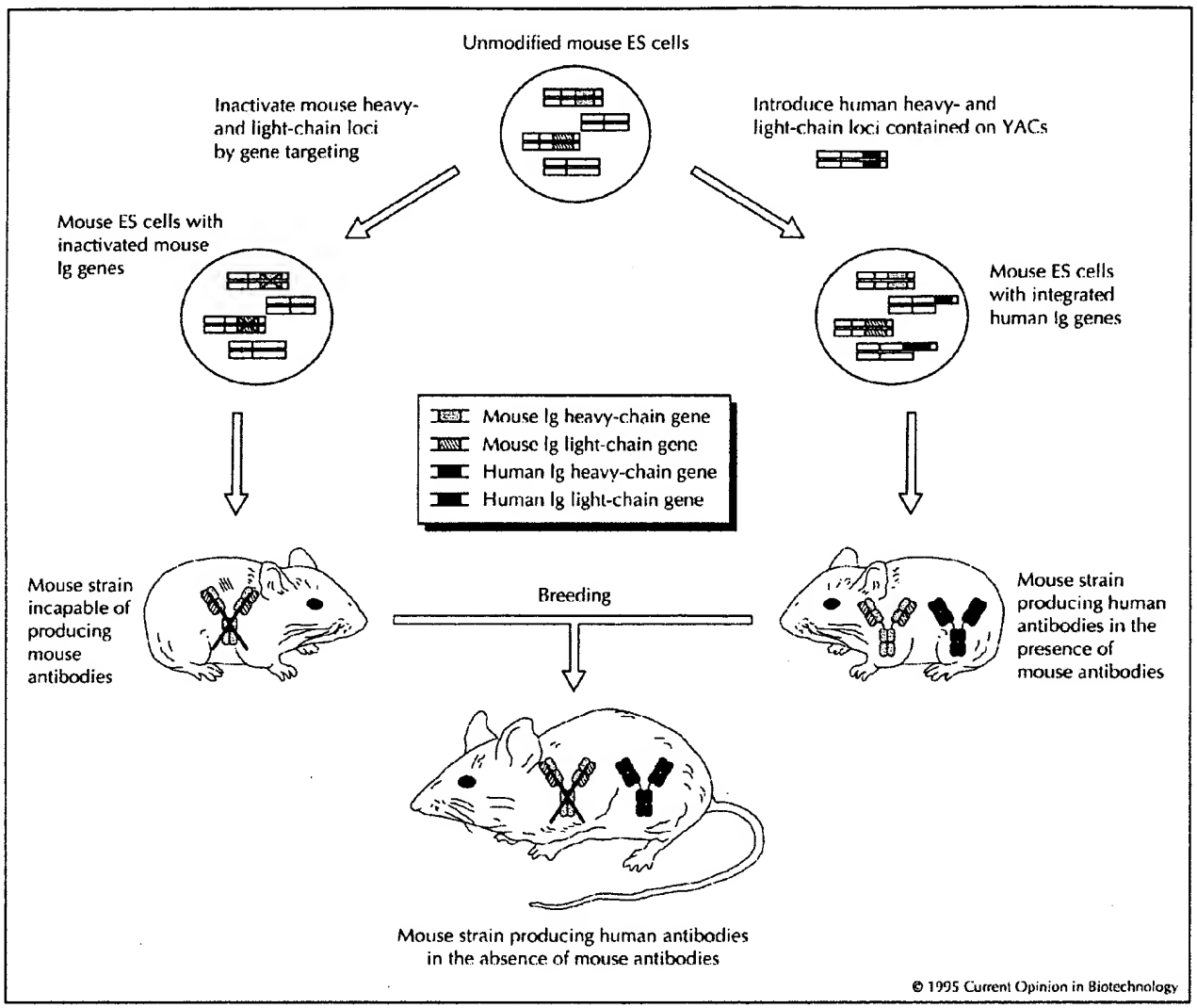

Fig. 1. An outline of the strategy used to generate xenomouse strains producing human antibodies in the absence of mouse antibodies [13••]. Mouse heavy-chain and κ light chain genes are inactivated in ES cells by gene-targeted deletion of J_H or C_κ, respectively. The targeted ES cells are utilized to generate mice homozygous for each of the J_H or C_κ deletions, rendering them incapable of producing mouse antibodies. Human heavy-chain and κ light chain fragments, contained on YACs, are introduced into ES cells via their fusion with yeast spheroplasts. The modified ES cells are used to generate mice that have integrated human Ig YACs and produce fully human antibodies in the presence of mouse antibodies. Breeding these mice with Ig-inactivated mice generates xenomouse strains.

mouse strains generated from gene targeted J_H and J_κ-C_κ ES cells to generate human antibody producing mice [21••].

Function of human immunoglobulin loci in mice

The human Ig genes have proven to be both compatible with the mouse system for rearrangement and expression and capable of substituting for the inactivated endogenous mouse Ig genes [13••,21••]. This has been demonstrated by their ability to restore B-cell development in Ig-inactivated mice and to induce significant production of fully human antibodies [13••,21••]. Human Ig YACs have been productively rearranged and expressed in mice, irrespective of their integration sites and copy number [13••]. Higher dependency on position and copy number has been observed in the expression of human Ig minigenes in mice, an approach requiring the generation of founder mice containing multiple copies [20,21••]. Decreased susceptibility to the influence of adjacent genomic sequences of human Ig YACs in mice may reflect their greater size and structural integrity and/or the presence of unidentified, but important, regulatory elements needed for optimal expression of the human Ig genes and their correct regulation.

Mature $B220^+$ cells have been readily detected in different lymphoid organs (i.e. bone marrow, spleen, peripheral blood, lymph nodes and peritoneum) from human Ig-bearing mice. All $B220^+$ cells express human heavy chain, and the majority of the cells co-express

exclusively human κ chain [13••,21••]. This population, in which the mouse lambda chain is absent, produces exclusively fully human antibodies. These results indicate that the human heavy chain can assemble properly with mouse surrogate light chains, mouse B-cell receptor components and the human light chain, allowing the development of mature functional B cells that express human antibodies.

Incomplete B-cell reconstitution, manifested in a partial block in pro- to pre-B-cell transition in bone marrow and in lower levels of mature B cells in lymphoid organs, has been observed in human Ig transgenic mice [13••,21••]. This may reflect an inefficient maturation of bone marrow derived B cells at the stage of VDJ rearrangement, which could be attributed to the limited V gene repertoire, and thus might suggest the value of introducing a greater number of human V genes.

Human antibody repertoire in human immunoglobulin transgenic mice

The nature of the human antibody repertoire directed by human Ig genes has been evaluated by analysis of heavy- and κ-chain transcripts for diversity of VDJ joinings and addition of non-germline nucleotides (N-additions). In xenomouse-derived transcripts, all J_H and J_K segments were represented at a frequency similar to that detected in adult human B cells [22]. High diversity of V and D segment usage is observed, with no position-biased utilization (i.e. D_{HQ52} and V_{VI}), reminiscent of adult human B cells. Frequent N segment addition was observed at V–D and D–J junctions, with an average length of 6.1 nucleotides compared with 7.7 nucleotides in adult human B cells [22]. The length of human heavy chain complementary determining region (CDR)-3 varied from 10 to 18 amino acids, with an average length of 12 amino acids. CDR3 sequences for κ chain transcripts were 9–10 amino acids in length. The features of a human Ig YAC-derived repertoire all appear to mirror the diversity seen in adult humans.

In transgenic mice with HC2 and KCo4 Ig minigenes [21••,23] or the shorter minigene constructs, HC1 (60 kb, two V_H genes) and Kc01 (25 kb, one V_K) [20,24], a diverse usage of J_H and J_K segments, similar to that of human B cells, is observed. In addition, utilization of the available V_H and V_K genes and different D segments is detected; however, a preponderance (~40%) of D_{HQ52} has been noted [20,23,24]. N-additions are observed at VDJ joins, with an average length of 3.8 nucleotides, generating heavy-chain CDR3 with an average length of 10.7 amino acids [20]. The position-biased usage of D segments and short N additions observed in these mice is characteristic of human fetal development [20,24]. The differences between the human antibody repertoire in mice bearing human Ig YACs and human Ig minigenes may reflect the greater size of the YAC and its germline configuration structure which may contain elements necessary to produce a normal adult human repertoire in the mouse.

Producing antigen-specific fully human antibodies in human immunoglobulin transgenic mice

Predominant expression of fully human antibodies, at levels of several 100 $\mu g l^{-1}$, has been demonstrated in sera of xenomouse strains and human Ig minigene bearing mice [13••,21••]. Low levels of hμ/mλ antibodies have also been detected. This level of fully human antibodies is several 100-fold higher than that detected in wild-type mice expressing human Ig genes [13••,20,21••], confirming the importance of inactivating mouse Ig genes to greatly increase human antibody production by mice.

Human γ1 antibodies have been detected in human Ig minigene containing mice, albeit at low levels, indicating class switching between the transgene μ and γ1 switch regions. Upon immunization, multiple non-germline-encoded nucleotides in human γ-derived variable regions are detected, suggesting that the somatic hypermutation process follows antigenic challenge [21••]. Human Ig transgenic mice are capable of mounting an antigen-specific human antibody response upon immunization with model antigens, such as tetanus toxin C, and human antigens, such as CD4 and IgE antibody [13••,21••]. In mice containing the human γ1 constant region, an initial antigen-specific human IgM response, followed by human IgG response has been detected, demonstrating a typical secondary immune response [21••]. Using conventional hybridoma technology, the immunized mice have been used successfully to generate mouse hybridomas producing antigen-specific human monoclonal antibodies [13••,21••]. An affinity of 9×10^7 M^{-1} has been demonstrated for the generated anti-human CD4 human antibodies [21••].

Conclusions

Introduction of human heavy-chain and κ light chain Ig fragments into the germline of Ig-inactivated mice generates mouse strains capable of producing authentic fully human antibodies. The human Ig genes are functional in the context of the mouse system for correct antibody recombination and expression, leading to induction of mouse B-cell development and the production of significant levels of a diverse repertoire of fully human antibodies. These mice are capable of mounting a human antibody response to human antigens, and they generate antigen-specific human antibodies whose diversity is created by VDJ recombination, junctional diversity and somatic hypermutation.

The success of large germline configuration human Ig YACs in exploiting the mouse system and eliciting a human adult-like repertoire suggests that introduction of even larger portions of the human heavy- and light-chain loci will result in reproduction of the human antibody response to infection and immunization. Delivery of megabase Ig gene fragments, which should be possible using the ES cell–yeast spheroplast fusion technology [15], would enable the introduction of a larger number of heavy- and light-chain variable genes, multiple heavy-chain constant regions and additional regulatory elements. The larger diversity of variable genes is likely to increase the level of B-cell reconstitution and to enhance the ability of the mouse to generate human antibodies with high affinity and specificity against any antigenic determinant. The availability of different γ constant regions will allow the generation of human antibodies with different effector functions as well as supporting optimal antibody affinity maturation. The additional control elements contained on these germline configuration fragments may contribute to even higher expression levels, optimal class switching, somatic hypermutation and allelic exclusion. Mouse strains containing these megabase Ig YACs may provide the optimal source for derivation of therapeutic human monoclonal antibodies against very broad spectrum human antigens and with any desired specificity. In addition, these strains may prove valuable for studying the molecular mechanisms and regulatory sequences influencing the programmed assembly and expression of human antibodies in the normal immune response, as well as the abnormal response characteristic of autoimmune disease and other disorders.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Kohler G, Milstein C: **Continuous cultures of fused cells secreting antibody of redefined specificity.** *Nature* 1975, 256:495–497.

2. Winter G, Milstein C: **Man-made antibodies.** *Nature* 1991, 349:293–299.

3. Morrison SL, Oi VT: **Chimeric immunoglobulin genes.** In *Immunoglobulin Genes.* Edited by Honjo T, Alt FW, Rabbitts TH. London: Academic Press; 1989:260–274.

4. Riechmann L, Clark M, Waldmann H, Winter G: **Reshaping human antibodies for therapy.** *Nature* 1988, 332:323–327.

5. Griffiths AD, Hoogenboom HR: **Building an *in vitro* immune system: human antibodies from phage display libraries.** In *Protein Engineering of Antibody Molecules for Prophylactic and Therapeutic Applications in Man.* Edited by Clark M. Nottingham: Academic Titles; 1993:45–64.

6. Burton DR, Barbas CF III: **Human antibodies from combinatorial libraries.** In *Protein Engineering of Antibody Molecules for Prophylactic and Therapeutic Applications in Man.* Edited by Clark M. Nottingham: Academic Titles; 1993:65–82.

7. Sandau J, Shpitz B, Gallinger S, Hozumi N: **Human primary immune response in SCID mice engrafted with human peripheral blood lymphocytes.** *J Immunol* 1994, 3806–3813.

8. Marcus H, David M, Canaan A, Kulova L, Lubin I, Segall H, Denes L, Erlich P, Galun E, Gan J *et al.*: **Human/mouse radiation chimeras are capable of mounting a human primary humoral response.** *Blood* 1995, in press.

9. Shin EK, Matsuda F, Nagaoka H, Fukita Y, Imai T, Yokoyama K, Soeda E, Honjo T: **Physical map of the 3′ region of the human immunoglobulin heavy chain locus: clustering of autoantibody-related variable segments in one haplotype.** *EMBO J* 1991, 10:3641–3645.

10. Weichhold GM, Ohnheiser R, Zachau HG: **The human immunoglobulin κ locus consists of two copies that are organized in opposite polarity.** *Genomics,* 1993, 16:503–511.

11. Cook GP, Tomlinson IM: **The human immunoglobulin V_H repertoire.** *Immunol Today* 1995, 16:237–242.

12. Max E: **Molecular genetics of immunoglobulins.** In *Fundamental Immunology.* Edited by Paul WE. New York: Raven Press; 1993:315–382.

13. •• Green LL, Hardy MH, Maynard-Currie CE, Tsuda H, Louie DM, Mendez MJ, Abderrahim H, Noguchi M, Smith DH, Zeng Y *et al.*: **Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs.** *Nature Genet* 1994, 17:13–21.

This paper describes a novel strategy for generating human antibody-producing mice by introduction of large fragments of human heavy- and κ-chain loci, contained on YACs, into the germline of Ig-inactivated mice. Such mice produce a diverse adult-like repertoire of fully human antibodies, and upon immunization, generate antigen-specific human monoclonal antibodies.

14. Burke DT, Olson MV: **Preparation of clone libraries in yeast artificial chromosome vectors.** *Methods Enzymol* 1991, 194:251–270.

15. Jakobovits A, Moore AL, Green LL, Vergara GJ, Maynard-Currie CE, Austin HA, Klapholz S: **Germ-line transmission and expression of a human-derived yeast artificial chromosome.** *Nature* 1993, 362:255–258.

16. • Mendez MJ, Abderrahim H, Noguchi M, David NE, Hardy MH, Green LL, Tsuda H, Yoast S, Maynard-Currie CE, Garza D *et al.*: **Analysis of the structural integrity of YACs comprising human immunoglobulin genes in yeast and in embryonic stem cells.** *Genomics* 1995, 26:294–307.

The report describes the cloning of human heavy- and κ-chain Ig YACs and the analysis of the integrity and stability of these YACs in yeast and upon introduction into ES cells

17. Jakobovits A, Vergara GJ, Kennedy JL, Hales JF, McGuinness RP, Casentini-Borocz DE, Brenner DG, Otten GR: **Analysis of homozygous mutant chimeric mice: deletion of the immunoglobulin heavy-chain joining region blocks B-cell development and antibody production.** *Proc Natl Acad Sci USA* 1993, 90:2551–2555.

18. Bruggemann M, Caskey HM, Teale C, Waldmann H, Williams GT, Surani MA, Neuberger MS: **A repertoire of monoclonal antibodies with human heavy chains from transgenic mice.** *Proc Natl Acad Sci USA* 1989, 86:6709–6713.

19. Wagner SD, Popov AV, Davies SL, Xian J, Neuberger MS, Bruggemann M: **The diversity of antigen-specific monoclonal antibodies from transgenic mice bearing human immunoglobulin gene miniloci.** *Eur J Immunol* 1994, 42:2672–2681.

20. Taylor LD, Carmack CE, Schramm S R, Mashayekh KM, Higgins KM, Kuo C-C, Woodhouse CS, Kay RM, Lonberg N: **A transgenic mouse that expresses a diversity of human sequence heavy and light chain immunoglobulins.** *Nucleic Acids Res* 1992, **20**:6287–6295.

21. •• Lonberg N, Taylor LD, Harding FA, Trounstine M, Higgins KM, Schramm SR, Kuo C-C, Mashayekh R, Wymore K, MaCabe JG *et al.*: **Antigen-specific human antibodies from mice comprising four distinct genetic modifications.** *Nature* 1994, **368**:856–859.
Human antibody-producing mouse strains are generated by introduction of human heavy- and κ-chain minigene constructs into the germline of Ig-inactivated mice. The production of human antibodies and antigen-specific human monoclonal antibodies is demonstarted.

22. Yamada M, Wasserman R, Reichard BA, Shane S, Caton AJ, Rovera G: **Preferential utilization of specific immunoglobulin heavy chain diversity and joining segments in adult human peripheral blood B lymphocytes.** *J Exp Med* 1991, **173**:395–407.

23. Taylor LD, Carmack CE, Huszar D, Higgins KM, Mashayekh R, Sequar G, Schramm SR, Kuo C-C, O'Donnel SL, Kay RM *et al.*: **Human immunoglobulin transgenes undergo rearrangement, somatic mutation and class switching in mice that lack endogenous IgM.** *Int Immunol* 1994, **6**:579–591.

24. Tuaillon N, Taylor LD, Lonberg N, Tucker PW, Capra JD: **Human immunoglobulin heavy-chain minilocus recombination in transgenic mice: gene-segment use in μ and γ transcripts.** *Proc Natl Acad Sci USA* 1993, **90**:3720–3724.

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